scientific reports

Check for updates

OPEN

Modulation of MHC expression by interferon-gamma and its influence on PBMC-mediated cytotoxicity in canine mast cell tumour cells

Sudchaya Bhanpattanakul[1], Theerawat Tharasanit[2,3], Supranee Buranapraditkun[4,5], Achariya Sailasuta[1], Takayuki Nakagawa[6] & Theerayuth Kaewamatawong[1,7]✉

Immunotherapy is a promising alternative treatment for canine mast cell tumour (MCT). However, evasion of immune recognition by downregulating major histocompatibility complex (MHC) molecules might decline treatment efficiency. Enhancing MHC expression through interferon-gamma (IFN-γ) is crucial for effective immunotherapy. In-house and reference canine MCT cell lines derived from different tissue origins were used. The impacts of IFN-γ treatment on cell viability, expression levels of MHC molecules, as well as cell apoptosis were evaluated through the MTT assay, RT-qPCR and flow cytometry. The results revealed that IFN-γ treatment significantly influenced the viability of canine MCT cell lines, with varying responses observed among different cell lines. Notably, IFN-γ treatment increased the expression of MHC I and MHC II, potentially enhancing immune recognition and MCT cell clearance. Flow cytometry analysis in PBMCs-mediated cytotoxicity assays showed no significant differences in overall apoptosis between IFN-γ treated and untreated canine MCT cell lines across various target-to-effector ratios. However, a trend towards higher percentages of late and total apoptotic cells was observed in the IFN-γ treated C18 and CMMC cell lines, but not in the VIMC and CoMS cell lines. These results indicate a variable response to IFN-γ treatment among different canine MCT cell lines. In summary, our study suggests IFN-γ's potential therapeutic role in enhancing immune recognition and clearance of MCT cells by upregulating MHC expression and possibly promoting apoptosis, despite variable responses across different cell lines. Further investigations are necessary to elucidate the underlying mechanisms and evaluate IFN-γ's efficacy in in vivo models.

Canine mast cell tumours (MCTs) are some of the most common malignant tumours in dogs[1,2]. Whilst surgery remains the primary treatment for MCTs, it may prove inadequate, particularly in malignant cases that require extensive surgical removal[3,4]. Chemotherapy and radiation therapy have also been used, but their effectiveness is limited. Moreover, the side effects of these treatments can significantly impact the quality of life of the animal[5–9]. Immunotherapy has emerged as a promising alternative treatment aimed to stimulate immune responses against tumours. Recognising the antigens presented by major histocompatibility complex (MHC) molecules on the tumour cell surface is one of the important immunological processes that can recognise and eliminate tumour

[1]Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. [2]Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. [3]Veterinary Clinical Stem Cells and Bioengineering Research Unit, Chulalongkorn University, Bangkok, Thailand. [4]Division of Allergy and Clinical Immunology, Department of Medicine, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Thai Red Cross Society, Bangkok 10330, Thailand. [5]Center of Excellence in Thai Pediatric Gastroenterology, Hepatology and Immunology (TPGHAI), Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. [6]Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. [7]Center of Excellence for Companion Animal Cancer (CE-CAC), Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. ✉email: theerayuth.k@chula.ac.th

cells. However, several tumours often evade the immune system's recognition by downregulating MHC expression or using other immune evasion strategies[10–12].

The relationship between MHC expression and tumour progression remains controversial and depends on various factors such as species and tumour types. Several studies revealed that the downregulation or loss of MHC expression in metastatic melanoma, triple-negative breast cancer and biliary tract cancer is associated with tumour progression and poor prognoses[13–15]. By contrast, research in human breast cancer and non-small cell lung cancer (NSCLC) demonstrated that a higher MHC expression is correlated with shorter overall survival rates[16,17]. For canine tumours, low MHC expression has been associated with the progression phase of canine transmissible venereal tumour (CTVT)[18], poor prognosis in B cell lymphoma[19] and mammary gland carcinomas[20].

Cancer immunotherapies including chimeric antigen receptor (CAR)-T cell therapy, immune checkpoint inhibitors and cytokine therapy are aimed at strengthening the immune system's ability to recognise tumours and restore immune effector function[21–24]. Interferon-gamma (IFN-γ) is a well-studied immune substance with potent anti-tumour effects, including inducing tumour cell cytotoxicity, increasing MHC-mediated antigen presentation and enhancing tumour recognition by antigen-specific $CD8^+$ and $CD4^+$ T lymphocyte[25–27]. The IFN-γ upregulates MHC expression through the JAK-STAT signalling pathway, involving the key transcriptional regulators NLR caspase recruitment domain-containing protein 5 (NLRC5) and class II transactivator (CIITA) for MHC class I (MHC I) and class II (MHC II), respectively[28–30]. Whilst IFN-γ has demonstrated growth-inhibitory effects on various canine tumour cell lines, including MCTs, mammary gland tumours and lymphoma cell lines, through cell cycle arrest[31], its specific effects on MHC expression in canine MCTs and the interactions with immune cells such as lymphocytes and natural killer (NK) cells are not well understood. To address this knowledge gap, we developed an in vitro co-culture model using IFN-γ-treated and untreated canine MCT cells with peripheral blood mononuclear cells (PBMCs) to investigate their immunological interactions and gain insights into the ex vivo immune response. Further research is required to deepen our understanding of these interactions and explore potential therapeutic approaches for managing canine MCTs.

This study aimed at optimising in vitro conditions to enhance MHC expression and investigating the cellular responses of canine MCT cell lines following treatment by IFN-γ in an MCT-PBMCs co-culture system.

Results

Effect of IFN-γ on the viability of canine MCT cell lines

In this study, we evaluated the effect of IFN-γ on the viability of canine MCT cell lines. The cells varied among cell lines after treatment with different concentrations of IFN-γ. As illustrated in Fig. 1, most of the MCT cell lines displayed resistance to IFN-γ, even at the highest concentrations (100 IU/mL), when compared to the control group. However, CMMC, VIMC and CoMS exhibited an increased cell viability in almost all conditions, whereas a slightly decreased in cell viability was observed in 10 and 100 IU/mL of IFN-γ-treated CMMC and 1 IU/mL of IFN-γ-treated VIMC and CoMS. Interestingly, the C18 cell line displayed a greater decrease in cell viability than the other cell lines after being exposed to IFN-γ treatment for 48 h.

Effect of IFN-γ on the expression of MHC I, MHC II and MHC transregulator genes

The relative mRNA expression levels of *NLRC5*, *MHC I*, *CIITA* and *MHC II* genes were quantitatively assessed at three time points (24, 48 and 72 h) following IFN-γ treatment. The results revealed variations in the responsiveness to IFN-γ among the different canine MCT cell lines (Fig. 2). A significant upregulation of these genes was observed at 24 and 48 h, indicating a positive response to IFN-γ stimulation. However, at 72 h, the expression levels showed a gradual decline in most cell lines. It is worth noting that the expression patterns of *NLRC5* and *CIITA* in the C18 cell line, as well as that of *MHC II* in the CMMC cell line treated with IFN-γ at a concentration of 100 IU/mL, deviated from the general trend observed for the other cell lines.

IFN-γ induced MHC I and MHC II expression at the cell surface level

Flow cytometry analysis was employed to investigate the expression of MHC I and MHC II on the cell surface, and the obtained results were consistent with the findings from RT-qPCR. The expression levels of MHC I and MHC II were categorised into four groups: cells that did not express either MHC I or MHC II ($MHC\ I^-/II^-$), cells expressing only MHC I ($MHC\ I^+/II^-$), cells expressing only MHC II ($MHC\ I^-/II^+$) and cells expressing both MHC I and MHC II ($MHC\ I^+/II^+$) (Fig. 3). Among the cell lines, the C18 cell line exhibited the highest proportion of cells expressing $MHC\ I^+/II^+$ both before and after treatment with IFN-γ. In the absence of treatment, the CMMC cell line had the highest proportion of $MHC\ I^-/II^+$ expression, followed by $MHC\ I^-/II^-$, $MHC\ I^+/II^+$ and $MHC\ I^+/II^-$, respectively. Conversely, the untreated CoMS and VIMC cell lines predominantly showed $MHC\ I^-/II^+$ expression, followed by $MHC\ I^+/II^+$, $MHC\ I^-/II^-$ and $MHC\ I^+/II^-$, respectively. After IFN-γ treatment, there was a notable increase in the proportion of cells expressing $MHC\ I^+/II^+$ in the CMMC, VIMC and CoMS cell lines.

After analysing the results from RT-qPCR and flow cytometry, the optimal condition to enhance MHC expression in the co-culture experiment was determined to be IFN-γ treatment at a concentration of 10 IU/mL for 48 h. Under this condition, the percentages of $MHC\ I^+$ and $MHC\ II^+$ expression were as follows: C18 (100.00% ± 0.00% and 100.00% ± 0.01%), CMMC (43.67% ± 0.14% and 94.64% ± 0.29%), VIMC (7.16% ± 1.18% and 92.87% ± 0.20%) and CoMS (29.25% ± 5.14% and 98.01% ± 1.32%) (see the statistical analysis data in Supplementary File 2 Table 1 and gating strategy in Supplementary Information, Fig. S1).

Effect of IFN-γ on apoptosis in canine MCT cell lines during co-culture with PBMCs

In the PBMCs-mediated cytotoxicity assay, flow cytometry analysis was performed to evaluate the populations of early, late, and total apoptotic cells in all canine MCT cell lines after treatment with IFN-γ (Fig. 4). The analysis

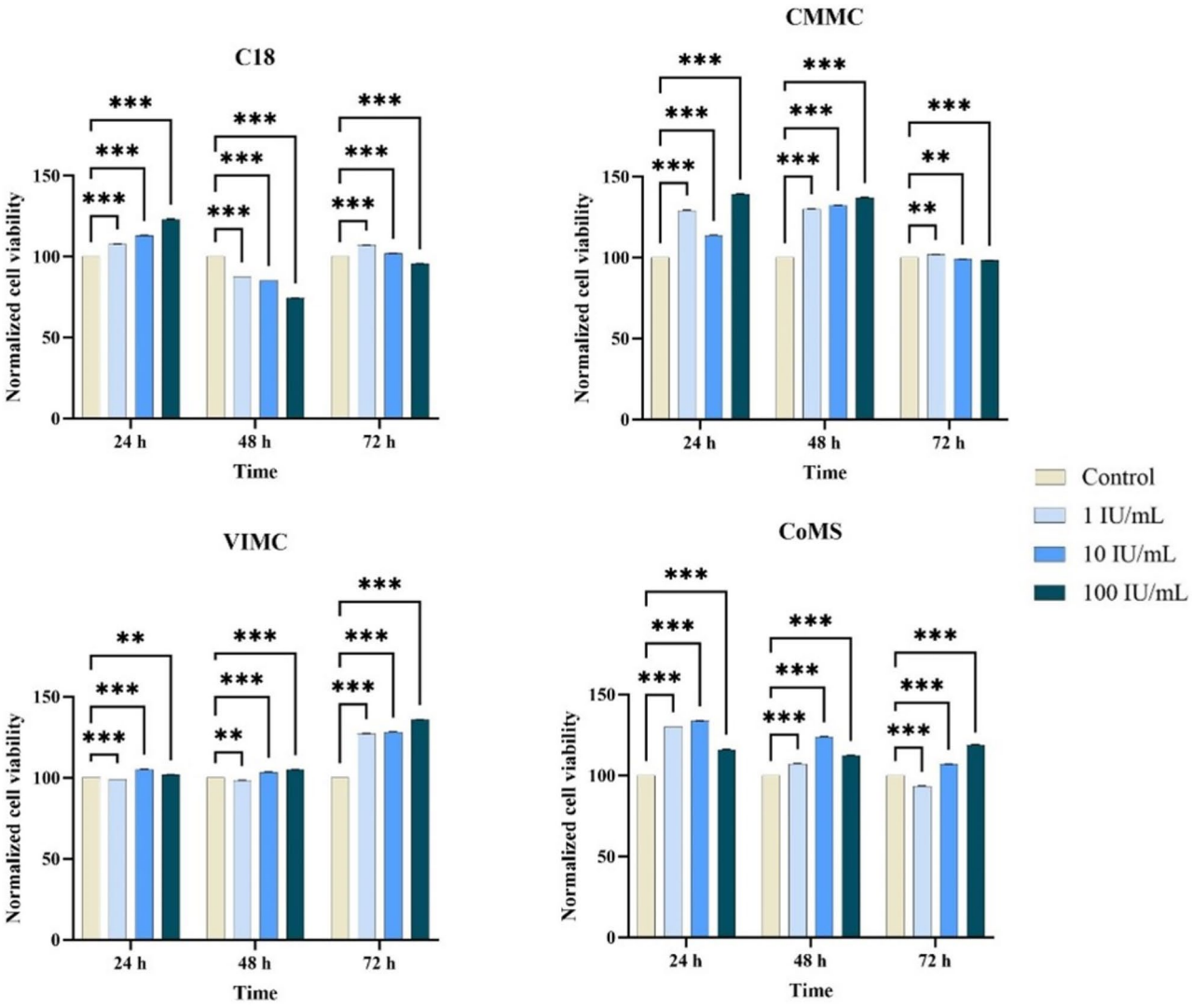

Figure 1. Cell viability after IFN-γ treatment was determined using the MTT colorimetric assay at 24-, 48-, and 72 h post-treatment (hpt). The results presented the percentage of viable cells compared to the control group, with error bars representing the standard deviation. The mean was calculated from three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

included various target-to-effector ratios (MCT cells to PBMCs: 1:25, 1:50, and 1:100) to comprehensively assess the impact of IFN-γ across different experimental conditions. The statistical analysis showed no significant differences in overall apoptosis between the treated and untreated groups across all ratios, indicating that IFN-γ treatment did not universally enhance apoptotic cell death in these cell lines. However, the data showed that the percentages of late and total apoptotic cells in the IFN-γ treated groups tended to be higher than in the untreated groups in the C18 and CMMC cell lines. In contrast, the VIMC and CoMS cell lines did not exhibit this pattern. These observations indicate a variable response to IFN-γ treatment among the different canine MCT cell lines. The complete raw data for each cell line, including the detailed percentages of apoptotic cells at each target-to-effector ratio, were provided in the supplementary information.

Discussion

During tumour development, tumours employ various strategies to evade recognition and elimination by the immune system. One well-documented mechanism for immune escape is the loss of MHC molecules on the surface of tumour cells[32,33]. Notably, IFN-γ is a promising therapeutic agent for canine atopic dermatitis[34,35] and plays a significant role in cell-mediated immune responses, particularly in its pleiotropic effects on antigen presentation through MHC molecules. These effects include the direct upregulation of MHC expression and the enhancement of antigen processing and loading by upregulating key components involved in these processes, such as proteasomal subunits LMP2 and LMP7, transporters associated with antigen processing (TAP) proteins and the proteasome regulator[36,37]. Our study provides further evidence of the ability of IFN-γ to upregulate MHC expression in canine MCT cell lines, consistent with previous reports such as in mouse models of prostate cancer and devil facial tumour cells[38,39]. However, despite these potential benefits, a comprehensive understanding of

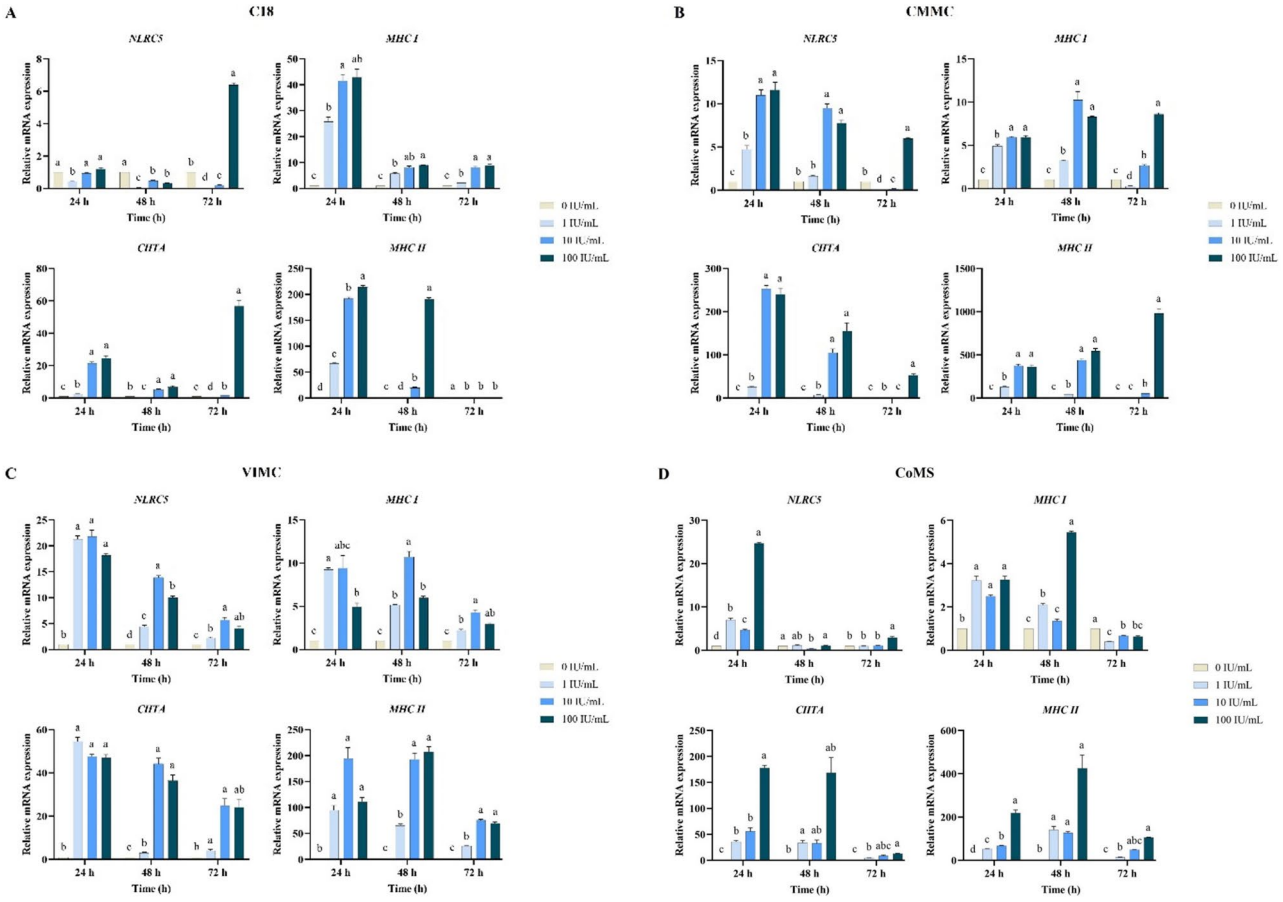

Figure 2. Effects of IFN-γ on the expression of MHC I, MHC II, and MHC Transregulator genes in canine MCT cell lines. RT-qPCR analysis was conducted to assess the relative mRNA expression levels of NLRC5, MHC I, CIITA, and MHC II genes at different time points following IFN-γ treatment in C18 (**A**), CMMC (**B**), VIMC (**C**), and CoMS (**D**) canine MCT cell lines.

the anti-tumour activity of IFN-, both in vitro and in vivo, is still lacking, necessitating controlled studies to assess its efficacy. Whilst large-scale clinical trials are essential for definitive evaluation, preliminary in vitro and in vivo investigations provide valuable insights into the underlying mechanisms of the anti-tumour activity of IFN-γ, contributing to a better understanding of its therapeutic potential.

In our study, we investigated the effect of IFN-γ treatment on the viability of canine MCT cell lines. Interestingly, most of the MCT cell lines exhibited resistance to IFN-γ treatment, even at high concentrations, which contrasts with a previous study reporting decreased cell proliferation in canine MCT cell lines upon IFN-γ treatment[31]. The underlying reasons for these varying sensitivities among cell lines were not elucidated in our study. Previous research suggests that defects in IFN-γ receptors and downstream signalling molecules, as well as differences in genetic makeup and physiological properties, may contribute to these variations in sensitivity across different cell lines[36,40,41]. Moreover, IFN-γ, known for its antiproliferative effects, can paradoxically promote cell viability and tumour progression through anti-apoptotic pathways and stemness induction. Variations in IFN-γ receptor (IFNγR) polymorphisms, as observed in IFNγR1-deficient HT-29 cells, suggest a role in regulating EGFR/Erk1/2 and Wnt/β-catenin pathways[42]. Human cancer cells such as DAMI, MDA-MD-468, and PC3 show that the C-terminal region of IFNγR2 contains a Bax inhibitory domain, suggesting resistance to apoptosis independent of JAK/STAT signaling[43]. Moreover, IFN-γ activates nNOS-NO signaling, leading to elevated NO levels, which can facilitate melanoma growth and aid immune evasion by upregulating PD-L1[44]. IFN-γ induces PD-L1 and PD-L2 expression for tumour immune evasion, thereby hindering T-cell responses[45,46]. Furthermore, low doses of IFN-γ activate ICAM1-PI3K-Akt-Notch1 signaling, enhancing CD133 expression and promoting cancer stemness in non-small cell lung cancer[47].

Furthermore, our investigation of MHC I and MHC II molecule expression revealed a significant increase in both gene and protein levels following IFN-γ treatment. However, we observed variations in the responsiveness to IFN-γ among the canine MCT cell lines, highlighting the complex and heterogeneous nature of the cellular response to this cytokine or potential alterations in the IFN-γ signalling pathway. Notably, MCT cell lines derived from mucosal origin exhibited lower MHC expression levels both before and after IFN-γ treatment, suggesting a potential loss of responsiveness to IFN-γ or dysfunction in the MHC antigen presentation pathway. These findings imply that these cell lines may be less susceptible to immune recognition and attack. Impairments in MHC

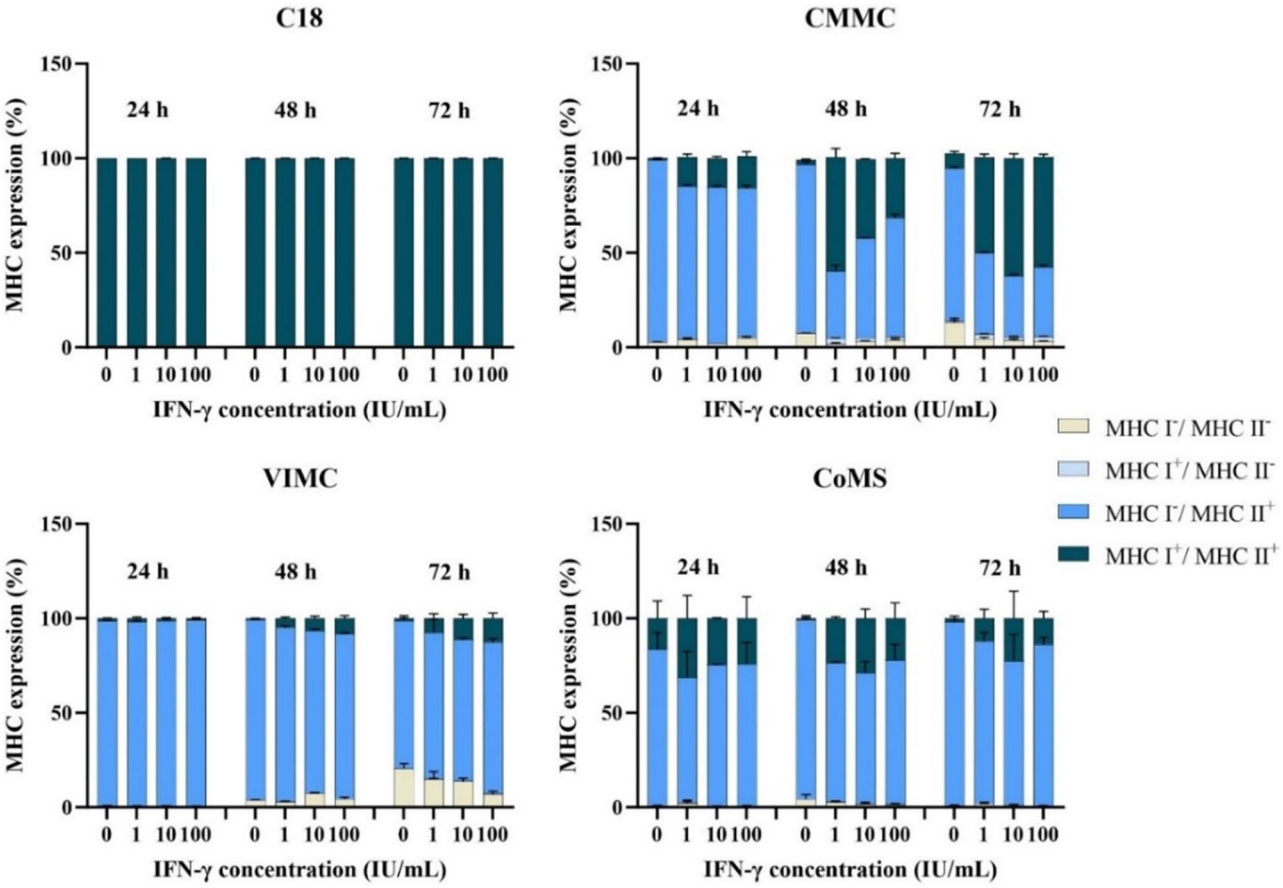

Figure 3. Effects of IFN-γ on MHC I and MHC II surface expression in canine MCT cell lines were evaluated by flow cytometry. The result is represented as a stacked bar graph for each cell line. Our findings indicate that CMMC, VIMC, and CoMS had a higher proportion of MHC I expression in all conditions after exposure to IFN-γ, in comparison to the control group. Notably, the C18 cell line displayed the highest proportion of MHC I+/II+ both before and after IFN-γ treatment.

antigen presentation can arise from defects in the antigen processing and presentation pathway or alterations in key components involved in MHC assembly and transport[33,37,38,48]. For instance, alterations in components such as tapasin, TAP, or β2-microglobulin can disrupt MHC I presentation, whereas mutations in the *CIITA* gene, responsible for regulating MHC II expression, can affect MHC II presentation[33,37,48–51].

Our findings suggest a potential application of IFN-γ as an anti-cancer agent for canine MCTs. While our study did not show a statistically significant difference in cell apoptosis between IFN-γ treated and untreated groups, we observed a trend where the percentages of late and total apoptotic cells were higher in the IFN-γ treated groups in the C18 and CMMC cell lines, both of which are of cutaneous origin. In contrast, the VIMC and CoMS cell lines, which are of mucosal origin, did not exhibit this pattern. These variations and lack of statistical significance may be due to heterogeneity in each cell line's response to PBMCs and the low sample size of the co-culture experiment in our study. This observation aligns with previous studies that have demonstrated the ability of PBMCs to induce cancer cell apoptosis through both intrinsic and extrinsic pathways. These pathways involve the release of perforin and granzyme B as well as the engagement of death receptors such as FasL/FasR and tumour necrosis factor (TNF)-α/TNFR1[52–57]. It's worth noting that IFN-γ can induce PD-L1 and PD-L2 expression, potentially facilitating tumour immune evasion[58–61]. In our research, we assessed PD-L1, PD-L2, and PD-1 expression in IFN-γ-treated MCT cell lines. While we observed increased expression levels, they were not statistically significant compared to untreated cells (detailed data are shown in Supplementary File 3). As for the results of PD-L1, PD-L2, and PD-1 expression, there was no statistically significant difference between the IFN-γ-treated and untreated groups. This suggests that these surface markers might not be involved in the tumour immunoregulatory mechanisms of PBMCs in IFN-γ-untreated and treated MCT in our study. Furthermore, our results indicate that treatment with IFN-γ enhances the expression of both MHC I and MHC II molecules in these cell lines, potentially promoting immune recognition and clearance of MCT cells by activated PBMCs. We also observed variations in the expression levels of MHC and the rates of apoptosis among different canine MCT cell lines, with cutaneous-origin cell lines exhibiting higher MHC expression and a greater propensity for apoptosis compared to mucosal-origin cell lines. These findings are consistent with the notion that canine mucosal MCTs have a more unfavourable prognosis compared to cutaneous MCTs, indicating potential differences in their biological characteristics and responses to therapeutic interventions[62]. The observed heterogeneity

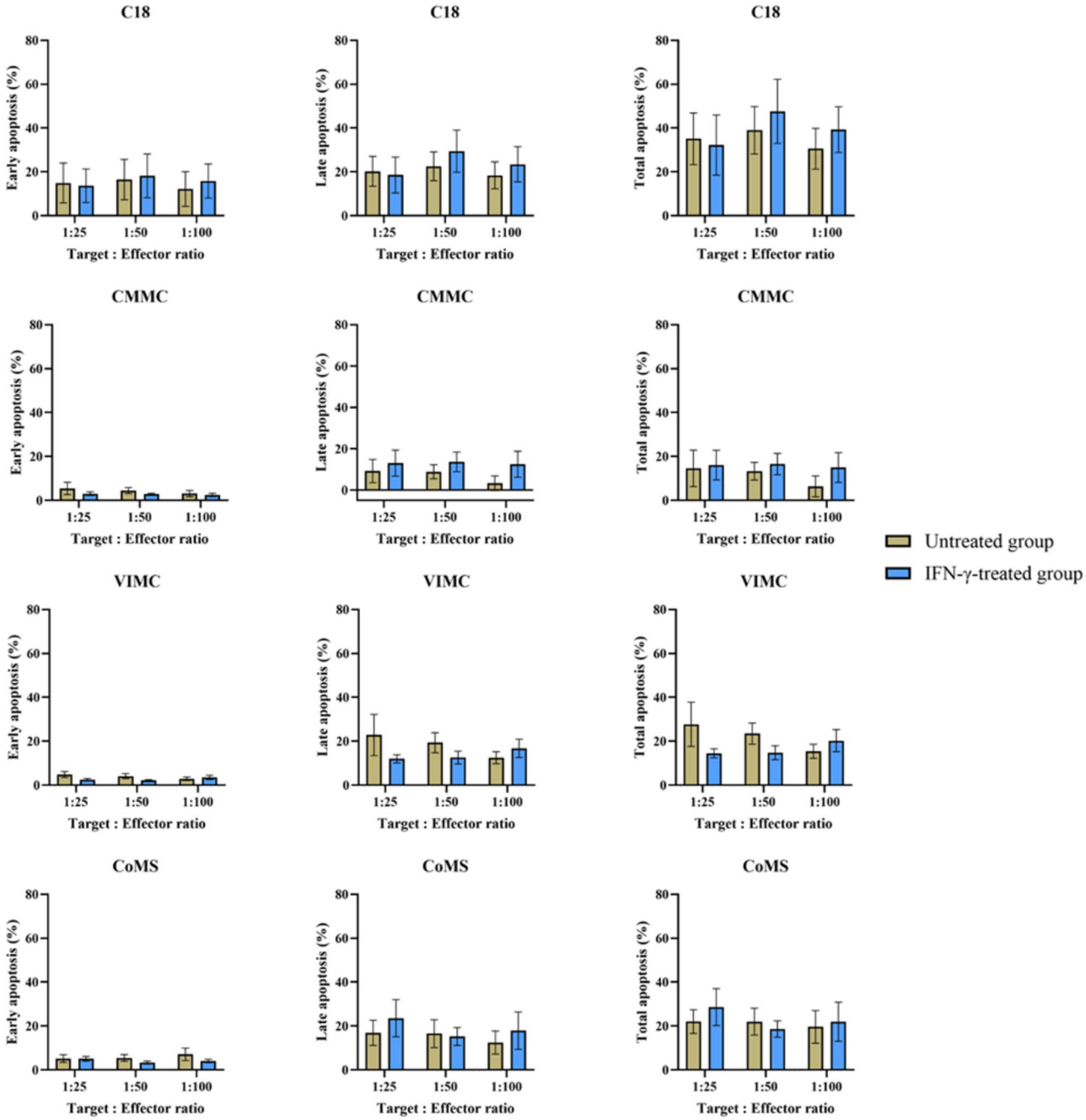

Figure 4. Comparison of the percentages of apoptotic cells between untreated and IFN-γ treated canine MCT cell lines across different target-to-effector ratios (MCT cells to PBMCs: 1:25, 1:50, and 1:100), evaluated using flow cytometry. The analysis showed no significant differences in apoptosis between the treated and untreated groups across all ratios. Additionally, the percentages of late and total apoptotic cells in the IFN-γ treated groups tended to be higher than in the untreated groups in the C18 and CMMC cell lines, in contrast to the VIMC and CoMS cell lines.

among MCT cells underscores the need for further investigations into their immunogenicity and susceptibility to treatments, such as IFN-γ-induced cytotoxicity, to develop effective therapeutic strategies.

Conclusions

In conclusion, our study provides valuable insights into the effects of IFN-γ on viability, MHC expression and apoptosis in canine MCT cell lines. The induction of apoptosis and upregulation of MHC expression support the potential therapeutic value of IFN-γ in the treatment of MCT in dogs. Further investigations with a good sample size are warranted to elucidate the underlying mechanisms of IFN-γ-induced apoptosis and to evaluate its efficacy in in vivo models. Understanding the interplay among IFN-γ, MHC antigen presentation and immune responses will contribute to the development of novel immunotherapeutic approaches for the treatment of canine MCTs.

Materials and methods

Experimental protocols

All procedures were performed according to protocols approved by Chulalongkorn University of Animal Care and Use (IACUC), Thailand (protocol no. 2131049), and Chulalongkorn University Faculty of Veterinary Science Biosafety Committee (CU-VET-BC), Thailand (protocol no. 2031029).

Cells and cell culture conditions

Four canine MCT cell lines (C18, CMMC, VIMC and CoMS) at passages 50–60 were used in this study. C18, an in-house established cell line, originates from a high-grade cutaneous MCT in a dog. A 13-year-old intact female Shih Tzu with a high-grade MCT in the right fourth mammary gland presented symptoms of depression, dehydration, and abdominal discomfort. Blood tests revealed anemia, leukocytosis, hypocalcemia, hyperphosphatemia, elevated ALP and BUN, and hypoproteinemia. Despite surgery, the dog succumbed 6 days later due to worsening condition. Histological analysis revealed an infiltrative subcutaneous MCT mass with densely packed, round cells displaying distinct borders, abundant basophilic granules, and moderate anisocytosis and anisokaryosis. Toluidine blue staining confirmed cell positivity. Cells were isolated using the tissue explant method and cultured in high-glucose DMEM (12800, Gibco) supplemented with 10% (v/v) heat-inactivated FBS (10270, Gibco), 1 mM L-glutamine (GlutaMAX™, 35050–061, Gibco), 1 mM antibiotic–antimycotic agents (Anti-Anti, 15240-062, Gibco), 1 mM nucleosides (EmbryoMax®, Sigma-Aldrich, USA), 1 mM MEM amino acids (M5550, Sigma-Aldrich, USA), and 1 mM non-essential amino acids (M7145, Sigma-Aldrich, USA). Cultures were maintained at 37 °C with 5% CO2, with medium changes every 3–4 days. In culture, cells typically exhibit a round shape, with anisocytosis and cell sizes ranging from 10 to 25 µm (Supplementary File 1, Fig. S1). This cell line can grow and proliferate as suspension cells without the need for supplementary growth factors. It has demonstrated remarkable longevity, sustaining cultivation for over 140 passages, equivalent to more than 1 year, with an average doubling time of 48.85 ± 0.86 h. Additionally, preliminary data indicate the expression of c-kit and tryptase at both the gene and protein levels, as assessed by RT-PCR and flow cytometry, respectively (S Bhanpattanakul, 2023, unpublished data). The other three reference cell lines were kindly provided by the Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, University of Tokyo. The CMMC originated from canine cutaneous MCT, while the VIMC and CoMS were derived from mucosal MCT[63,64]. All cell lines were propagated in a complete culture medium, which consisted of Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS, Gibco), 1% (v/v) antibiotic–antimycotic (Gibco) and 1% (v/v) L-glutamine (Gibco). The cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

Study of the effect of recombinant canine-interferon-gamma (IFN-γ) on canine MCT cell lines

Treatment of canine MCT cell lines with IFN-γ

Each canine MCT cell line was seeded at 2×10^5 cells/mL and treated with IFN-γ (781-CG-050, R&D Systems, USA) at a concentration of 0 (control group), 1, 10 and 100 IU/mL. After 24, 48 and 72 h of treatment, cells were harvested and evaluated for cell viability, expression of MHC-related genes and quantitative MHC expression. The appropriate IFN-γ concentration, which can enhance MHC I and MHC II expression in all cell lines, was chosen for the PBMC-mediated cytotoxicity test.

Evaluation of cell viability

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were pelleted, and 1 mL of 0.5 mg/mL MTT reagent (Invitrogen, USA) was added, followed by incubation for 2 h in a humidified atmosphere at 37 °C, 5% CO_2. Afterward, the solution was discarded, and 1 mL of dimethyl sulfoxide (DMSO; Thermo Fisher Scientific INC., USA) was added. After the cells and dye crystals were solubilised, absorbance was measured using a microplate reader (TECAN, HydroFlex™ Platform, Austria) at a wavelength of 570 nm. The results were reported as the mean percentage viability of the treated cells compared to that of the control group.

Determination of MHC I, MHC II and MHC transregulator gene expression

To explore the levels of MHC and MHC transregulator gene expression after IFN-γ treatment, quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed. The total RNA was extracted from cells in each condition using the RNeasy mini kit (Qiagen, Hilden, Germany) and processed following the manufacturer's instructions. The total RNA quantity was determined using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific INC., USA), and the RNA was immediately stored at -80 °C until use. The genomic DNA was treated with DNase I (Promega, WI, USA). The cDNA was synthesised using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA), and the qPCR was performed on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each qPCR reaction mix (15 µL) was comprised of KAPA SYBR® FAST qPCR Kits (KK4600, Kapa Biosystems, Woburn, MA, USA), 200 nM of each primer (final concentration) and 10 ng cDNA. Nuclease-free water was used as no-template control. Thermal cycling was performed as follows: Stage 1 at 50 °C for 2 min, Stage 2 at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s and 60 °C for 30 s, with annealing/extension at 72 °C for 1 min. All qPCR reactions were performed in triplicate in three independent runs. Data are reported as the relative expression difference between the IFN-γ-treatment and the control group. The relative expression levels of *NLRC5*, *MHC I*, *CIITA* and *MHC II* genes were normalised to a stable reference gene (*RPS5*). The specific primers are shown in Table 1.

Gene	Sequence (5′–3′)	Accession no.	Amplicon size (bp)
MHC I (DLA-88)	F: CCTGTCCCTAATCTGATGTG	NM_001014767.1	165
	R: AGACTCAGGAAACACAAACT		
NLRC5	F: TGTTCACTCAAGGACTAAGC	XM_014109337.2	225
	R: TCCCTGAGAGGTCTATCTTC		
MHC II (DLA-DRB1)	F: GTAATGGAGACTGGACCTTC	NM_001014768.1	237
	R: AGTGTCCCTTCTGATTTCTG		
CIITA	F: CTGAGTGAGAACAAGATCGG	XM_022420073.1	188
	R: ATGTAGTTATTGTACAGGCTCA		
RPS5	F: TCACTGGTGAGAACCCCCT	XM_533568.4	141
	R: CCTGATTCACACGGCGTAG		

Table 1. Canine-specific primer sequences used for the quantitative polymerase chain reaction. *MHC,* Major histocompatibility complex; *NLRC5,* NOD-, LRR- and CARD-containing 5; *CIITA*, Class II transactivator; *RPS5*, Ribosomal protein S5.

Analysis of MHC I and MHC II expression on the cell surface

For assessment of the MHC expression, cells of each condition were analysed using flow cytometry. Briefly, the cells were washed twice with phosphate-buffered saline (PBS) solution and blocked via non-specific binding using 0.1% bovine serum albumin (BSA) solution at 25 °C for 20 min. Subsequently, the cells were labelled with the primary antibodies anti-MHC I (mouse monoclonal, 1:200, DG-H58A, Novus Biologicals, USA) and anti-MHC II (rabbit polyclonal, 1:500, ab180779, Abcam, USA) at 4 °C overnight. Afterward, the cells were washed with PBS and incubated with the secondary antibodies: TRITC-conjugated goat anti-mouse IgG (1:500, T5393, Sigma-Aldrich, USA) and FITC-conjugated goat anti-rabbit IgG (1:500, 11-4839-81, Thermo Fisher Scientific INC., USA) at 37 °C for 1 h in the dark, followed by washing with PBS and fixation with 2% (w/v) paraformaldehyde. The MHC expression was analysed using a BD FACSCalibur flow cytometer (BD, USA) with the BD CellQuest™ Pro software. The results are shown as the percentages of MHC I- and/or MHC II-positive cells.

PBMCs-mediated cytotoxicity assay

Preparation of PBMCs

Peripheral blood samples were obtained from five healthy beagle dog donors. The samples were centrifuged at 3000 rpm at 25 °C for 10 min, and the supernatant was discarded. The cells were resuspended with 10 mL of culture medium, carefully layered onto 4 mL of Histopaque®-1077 (H8889, Sigma, USA) and centrifuged at 2200 rpm 25 °C for 20 min with the brake turned off. After centrifugation, the white layer of PBMCs at the interphase was gently harvested by aspiration using a Pasteur pipette and transferred to a new tube. Subsequently, the PBMCs were washed twice with 20 mL of culture medium and centrifuged at 2000 rpm at 25 °C for 10 min. The isolated PBMCs were cryopreserved at -80 °C until later use.

PBMC-mediated cytotoxicity assay

The isolated PBMCs were activated with mitomycin C-treated MCT cell lines (with or without IFN-γ treatment) for 14 days before cytotoxicity analysis. The isolated PBMCs were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS (10270, Gibco), 1 mM L-glutamine (GlutaMAX™, 35050-061, Gibco), 1 mM antibiotic–antimycotic agents (Anti-Anti, 15240-062, Gibco), and 100 U/mL of recombinant human interleukin-2 (rhIL-2). Half of the medium was discarded and replaced with fresh medium every 3 days. The diagram illustrating the activation of isolated PBMCs is provided in Supplementary Information, Fig. S2. For cytotoxicity testing, a total of 1×10^5 fresh IFN-γ-treated MCT cell lines (target cells) were seeded for 30 min, and subsequently, the PBMCs were co-cultured at the different target-to-effector (T: E) ratios (1:25, 1:50 and 1:100). A monoculture of MCT cells served as the control group. After 4 h of co-culture, the cells were collected and analysed for cell apoptosis by flow cytometry using the FITC Annexin V Apoptosis Detection Kit with propidium iodide (640914, BioLegend, San Diego, CA, USA). Briefly, cells were washed twice with cold BioLegend's cell staining buffer and resuspended in 100 µL of Annexin V binding buffer. Each sample was stained with 5 µL of FITC Annexin V and 10 µL of propidium iodide and incubated for 15 min at 25 °C in the dark. The Annexin V binding buffer was added to each sample to adjust the final volume to 500 µL. Subsequently, the samples were immediately analysed using a BD FACSCalibur flow cytometer (BD, USA) with the BD CellQuest™ Pro software.

Statistical analysis

The results are presented as mean ± standard error of the mean (SEM) from three independent experiments. Normal distribution was assessed by the Shapiro test. One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were used to determine differences between treatment groups. Differences between each time point were examined using repeated measures. The GraphPad Prism software version 9.5.1 was used for all statistical analyses; $P < 0.05$ was considered statistically significant.

Ethics declarations

All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Chulalongkorn University (protocol 2131049) which complied with the ARRIVE guidelines. (The full form of IACUC was in the supplementary document). The biosafety procedures followed the guidelines of the Institutional Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (No. IBC2031029).

Data availability

The data used in this study are available from the corresponding author upon reasonable request.

Received: 4 April 2024; Accepted: 29 July 2024
Published online: 01 August 2024

References

1. Baioni, E. *et al.* Estimating canine cancer incidence: Findings from a population-based tumour registry in northwestern Italy. *BMC Vet. Res.* **13**, 1–9 (2017).
2. Welle, M. M., Bley, C. R., Howard, J. & Rüfenacht, S. Canine mast cell tumours: A review of the pathogenesis, clinical features, pathology and treatment. *Vet. Dermatol.* **19**, 321–339 (2008).
3. Hahn, K. A. *et al.* Evaluation of 12- and 24-month survival rates after treatment with masitinib in dogs with nonresectable mast cell tumors. *Am. J. Vet. Res.* **71**, 1354–1361. https://doi.org/10.2460/ajvr.71.11.1354 (2010).
4. London, C. A. *et al.* Multi-center, placebo-controlled, double-blind, randomized study of oral toceranib phosphate (SU11654), a receptor tyrosine kinase inhibitor, for the treatment of dogs with recurrent (either local or distant) mast cell tumor following surgical excision. *Clin. Cancer Res.* **15**, 3856–3865. https://doi.org/10.1158/1078-0432.CCR-08-1860 (2009).
5. Kiupel, M. *et al.* Proposal of a 2-tier histologic grading system for canine cutaneous mast cell tumors to more accurately predict biological behavior. *Vet. Pathol.* **48**, 147–155. https://doi.org/10.1177/0300985810386469 (2011).
6. Moirano, S. J., Lima, S. F., Hume, K. R. & Brodsky, E. M. Association of prognostic features and treatment on survival time of dogs with systemic mastocytosis: A retrospective analysis of 40 dogs. *Vet. Comp. Oncol.* **16**, E194–E201. https://doi.org/10.1111/vco.12373 (2018).
7. Pizzoni, S. *et al.* Features and prognostic impact of distant metastases in 45 dogs with de novo stage IV cutaneous mast cell tumours: A prospective study. *Vet. Comp. Oncol.* **16**, 28–36. https://doi.org/10.1111/vco.12306 (2018).
8. Takeuchi, Y. *et al.* Validation of the prognostic value of histopathological grading or c-kit mutation in canine cutaneous mast cell tumours: A retrospective cohort study. *Vet. J.* **196**, 492–498 (2013).
9. Webster, J. D., Yuzbasiyan-Gurkan, V., Thamm, D. H., Hamilton, E. & Kiupel, M. Evaluation of prognostic markers for canine mast cell tumors treated with vinblastine and prednisone. *BMC Vet. Res.* **4**, 1–8 (2008).
10. Angell, T. E., Lechner, M. G., Jang, J. K., LoPresti, J. S. & Epstein, A. L. MHC class I loss is a frequent mechanism of immune escape in papillary thyroid cancer that is reversed by interferon and selumetinib treatment in vitro. *Clin. Cancer Res.* **20**, 6034–6044 (2014).
11. Gobbo, J. *et al.* Restoring anticancer immune response by targeting tumor-derived exosomes with a HSP70 peptide aptamer. *J. Natl. Cancer Inst.* **108**, djv330 (2016).
12. Westergaard, M. C. W. *et al.* Changes in the tumor immune microenvironment during disease progression in patients with ovarian cancer. *Cancers* **12**, 3828 (2020).
13. Carretero, R. *et al.* Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy. *Immunogenetics* **60**, 439–447. https://doi.org/10.1007/s00251-008-0303-5 (2008).
14. Forero, A. *et al.* Expression of the MHC class II pathway in triple-negative breast cancer tumor cells is associated with a good prognosis and infiltrating lymphocytes. *Cancer Immunol. Res.* **4**, 390–399. https://doi.org/10.1158/2326-6066.CIR-15-0243 (2016).
15. Goeppert, B. *et al.* Major histocompatibility complex class I expression impacts on patient survival and type and density of immune cells in biliary tract cancer. *Br. J. Cancer* **113**, 1343–1349. https://doi.org/10.1038/bjc.2015.337 (2015).
16. Madjd, Z., Spendlove, I., Pinder, S. E., Ellis, I. O. & Durrant, L. G. Total loss of MHC class I is an independent indicator of good prognosis in breast cancer. *Int. J. Cancer* **117**, 248–255 (2005).
17. Ramnath, N. *et al.* Is downregulation of MHC class I antigen expression in human non-small cell lung cancer associated with prolonged survival?. *Cancer Immunol. Immunother.* **55**, 891–899 (2006).
18. Hsiao, Y.-W., Liao, K.-W., Hung, S.-W. & Chu, R.-M. Effect of tumor infiltrating lymphocytes on the expression of MHC molecules in canine transmissible venereal tumor cells. *Vet. Immunol. Immunopathol.* **87**, 19–27 (2002).
19. Rao, S. *et al.* Class II major histocompatibility complex expression and cell size independently predict survival in canine B-cell lymphoma. *J. Vet. Intern. Med.* **25**, 1097–1105 (2011).
20. Tanaka, T. *et al.* Relationship between major histocompatibility complex class I expression and prognosis in canine mammary gland tumors. *J. Vet. Med. Sci.* **75**, 1393–1398 (2013).
21. Fesnak, A. D., June, C. H. & Levine, B. L. Engineered T cells: The promise and challenges of cancer immunotherapy. *Nat. Rev. Cancer* **16**, 566–581 (2016).
22. Finn, O. Immuno-oncology: Understanding the function and dysfunction of the immune system in cancer. *Ann. Oncol.* **23**, 6–9 (2012).
23. Smyth, M. J., Ngiow, S. F., Ribas, A. & Teng, M. W. Combination cancer immunotherapies tailored to the tumour microenvironment. *Nat. Rev. Clin. Oncol.* **13**, 143–158 (2016).
24. Yang, Y. Cancer immunotherapy: Harnessing the immune system to battle cancer. *J. Clin. Invest.* **125**, 3335–3337 (2015).
25. Alspach, E., Lussier, D. M. & Schreiber, R. D. Interferon γ and its important roles in promoting and inhibiting spontaneous and therapeutic cancer immunity. *Cold Spring Harb. Perspect. Biol.* **11**, a028480 (2019).
26. Zhao, M. *et al.* MHC class II transactivator (CIITA) expression is upregulated in multiple myeloma cells by IFN-γ. *Mol. Immunol.* **44**, 2923–2932 (2007).
27. Zhou, F. Molecular mechanisms of IFN-gamma to up-regulate MHC class I antigen processing and presentation. *Int. Rev. Immunol.* **28**, 239–260. https://doi.org/10.1080/08830180902978120 (2009).
28. Kobayashi, K. S. & van den Elsen, P. J. NLRC5: A key regulator of MHC class I-dependent immune responses. *Nat. Rev. Immunol.* **12**, 813–820. https://doi.org/10.1038/nri3339 (2012).
29. Meissner, T. B. *et al.* NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 13794–13799. https://doi.org/10.1073/pnas.1008684107 (2010).
30. Neerincx, A., Castro, W., Guarda, G. & Kufer, T. A. NLRC5, at the heart of antigen presentation. *Front. Immunol.* **4**, 397. https://doi.org/10.3389/fimmu.2013.00397 (2013).
31. Hamamura, Y. *et al.* The inhibitory effect of canine interferon gamma on the growth of canine tumors. *Res. Vet. Sci.* **132**, 466–473 (2020).

32. Algarra, I., García-Lora, A., Cabrera, T., Ruiz-Cabello, F. & Garrido, F. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: Implications for tumor immune escape. *Cancer Immunol. Immunother.* **53**, 904–910 (2004).
33. Garrido, F., Algarra, I. & García-Lora, A. M. The escape of cancer from T lymphocytes: Immunoselection of MHC class I loss variants harboring structural-irreversible "hard" lesions. *Cancer Immunol. Immunother.* **59**, 1601–1606 (2010).
34. Iwasaki, T. & Hasegawa, A. A randomized comparative clinical trial of recombinant canine interferon-γ (KT-100) in atopic dogs using antihistamine as control. *Vet. Dermatol.* **17**, 195–200 (2006).
35. Yasukawa, K. *et al.* Low-dose recombinant canine interferon-γ for treatment of canine atopic dermatitis: An open randomized comparative trial of two doses. *Vet. Dermatol.* **21**, 42–49 (2010).
36. Dunn, G. P., Koebel, C. M. & Schreiber, R. D. Interferons, immunity and cancer immunoediting. *Nat. Rev. Immunol.* **6**, 836–848. https://doi.org/10.1038/nri1961 (2006).
37. Seliger, B., Ruiz-Cabello, F. & Garrido, F. IFN inducibility of major histocompatibility antigens in tumors. *Adv. Cancer Res.* **101**, 249–276 (2008).
38. Martini, M. *et al.* IFN-gamma-mediated upmodulation of MHC class I expression activates tumor-specific immune response in a mouse model of prostate cancer. *Vaccine* **28**, 3548–3557. https://doi.org/10.1016/j.vaccine.2010.03.007 (2010).
39. Ong, C. E., Lyons, A. B., Woods, G. M. & Flies, A. S. Inducible IFN-γ expression for MHC-I upregulation in devil facial tumor cells. *Front. Immunol.* **9**, 3117 (2019).
40. Dunn, G. P. *et al.* Interferon-γ and cancer immunoediting. *Immunol. Res.* **32**, 231–245 (2005).
41. Kaplan, D. H. *et al.* Demonstration of an interferon γ-dependent tumor surveillance system in immunocompetent mice. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 7556–7561 (1998).
42. Wang, L., Wang, Y., Song, Z., Chu, J. & Qu, X. Deficiency of interferon-gamma or its receptor promotes colorectal cancer development. *J. Interferon Cytokine Res.* **35**, 273–280 (2015).
43. Gomez, J. A. *et al.* The C-terminus of interferon gamma receptor beta chain (IFNγR2) has antiapoptotic activity as a Bax inhibitor. *Cancer Biol. Therapy* **8**, 1771–1786 (2009).
44. Tong, S. *et al.* Inhibition of interferon-gamma-stimulated melanoma progression by targeting neuronal nitric oxide synthase (nNOS). *Sci. Rep.* **12**, 1701 (2022).
45. Heimes, A.-S. *et al.* Prognostic significance of interferon-γ and its signaling pathway in early breast cancer depends on the molecular subtypes. *Int. J. Mol. Sci.* **21**, 7178 (2020).
46. Martin, V. *et al.* Met inhibition revokes IFNγ-induction of PD-1 ligands in MET-amplified tumours. *Br. J. Cancer* **120**, 527–536 (2019).
47. Song, M. *et al.* Low-dose IFNγ induces tumor cell stemness in tumor microenvironment of non–small cell lung cancer. *Cancer Res.* **79**, 3737–3748 (2019).
48. Seliger, B. Molecular mechanisms of MHC class I abnormalities and APM components in human tumors. *Cancer Immunol. Immunother.* **57**, 1719–1726 (2008).
49. Bijen, C. B. *et al.* The prognostic role of classical and nonclassical MHC class I expression in endometrial cancer. *Int. J. Cancer* **126**, 1417–1427. https://doi.org/10.1002/ijc.24852 (2010).
50. Reith, W., LeibundGut-Landmann, S. & Waldburger, J. M. Regulation of MHC class II gene expression by the class II transactivator. *Nat. Rev. Immunol.* **5**, 793–806. https://doi.org/10.1038/nri1708 (2005).
51. Sanda, M. G. *et al.* Molecular characterization of defective antigen processing in human prostate cancer. *J. Natl. Cancer Inst.* **87**, 280–285 (1995).
52. Brennan, A., Chia, J., Trapani, J. & Voskoboinik, I. Perforin deficiency and susceptibility to cancer. *Cell Death Differ.* **17**, 607–615 (2010).
53. Messmer, M. N., Snyder, A. G. & Oberst, A. Comparing the effects of different cell death programs in tumor progression and immunotherapy. *Cell Death Differ.* **26**, 115–129 (2019).
54. Raskov, H., Orhan, A., Christensen, J. P. & Gögenur, I. Cytotoxic CD8+ T cells in cancer and cancer immunotherapy. *Br. J. Cancer* **124**, 359–367 (2021).
55. Russell, J. H. & Ley, T. J. Lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* **20**, 323 (2002).
56. Smyth, M. J. & Johnstone, R. W. Role of TNF in lymphocyte-mediated cytotoxicity. *Microsc. Res. Tech.* **50**, 196–208 (2000).
57. Voskoboinik, I., Smyth, M. J. & Trapani, J. A. Perforin-mediated target-cell death and immune homeostasis. *Nat. Rev. Immunol.* **6**, 940–952 (2006).
58. Velcheti, V. *et al.* Programmed death ligand-1 expression in non-small cell lung cancer. *Lab. Investig.* **94**, 107–116 (2014).
59. Jorgovanovic, D., Song, M., Wang, L. & Zhang, Y. Roles of IFN-γ in tumor progression and regression: A review. *Biomark. Res.* **8**, 1–16 (2020).
60. Abiko, K. *et al.* IFN-γ from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer. *Br. J. Cancer* **112**, 1501–1509 (2015).
61. Bellucci, R. *et al.* Interferon-γ-induced activation of JAK1 and JAK2 suppresses tumor cell susceptibility to NK cells through upregulation of PD-L1 expression. *Oncoimmunology* **4**, e1008824 (2015).
62. Takahashi, T. *et al.* Visceral mast cell tumors in dogs: 10 cases (1982–1997). *J. Am. Vet. Med. Assoc.* **216**, 222–226 (2000).
63. Ishiguro, T. *et al.* Establishment and characterization of a new canine mast cell tumor cell line. *J. Vet. Med. Sci.* **63**, 1031–1034 (2001).
64. Takahashi, T. *et al.* IgG-mediated histamine release from canine mastocytoma-derived cells. *Int. Arch. Allergy Immunol.* **125**, 228–235 (2001).

Acknowledgements

We would like to express our gratitude to the staff of the Department of Obstetrics, Gynaecology, and Reproduction at the Small Animal Teaching Hospital, Chulalongkorn University, Bangkok, Thailand, for their assistance with blood sample collection and valuable suggestions.

Author contributions

TT and TK conceptualised and supervised this study. SB performed the experiments. SB and SBu performed flow cytometry and analysed the data. TN and AS provided canine MCT cell lines. SB drafted the manuscript. TT and TK reviewed and edited the original draft. All authors read and approved the final manuscript.

Funding

This research was supported by the National Research Council of Thailand (N41D640002), the Chulalongkorn University Graduate Scholarship to the 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship, the 90th Anniversary of Chulalongkorn University, Rachadapisek Sompote Endowment Fund 2019 (CU_GR_62_77_31_07) and the Center of Excellence for Companion Animal Cancer (CE-CAC).

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-68789-7.

Correspondence and requests for materials should be addressed to T.K.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.